# AUTOPHAGY GENES (*ATG16L1* AND *IRGM*) VARIANTS AND HEPATITIS B VIRUS INFECTION SUSCEPTIBILITY

## *SYNOPSIS*

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## Introduction

Hepatitis is a liver infection caused by the hepatitis B virus and it represents a serious public health problem globally with significant morbidity and mortality [1]. Jaundice, abdominal pain, liver enlargement, muscles or joint pain, loss of appetite and fever, characterizes it. Hepatitis B virus (HBV) transmission occurs from direct contact with infected blood, parenterally via infected blood transfusions, intravenous drug abuse, vertically from an infected mother to child, horizontally (*e.g.* between children in a household) and via sexual routes [2]. The HBV can survive outside the body for at least 7 days and during this period; it is capable of causing infection. The average incubation period of hepatitis B virus is 75 days and can vary from 30-180 days [3]. According to World Health Organization estimate, worldwide around 257 million people are living with chronic HBV infection [3]. HBV leads to a wide range of liver diseases from acute to chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [4]. Individuals with chronic HBV infection are more prone to develop liver cirrhosis (LC) and hepatocellular carcinoma (HCC) and in most of Asia and Africa, HBV is the primary risk factor for the development of HCC [3].

India has one-fifth of the World's population and it carries the second largest burden of HBV [5]. According to the WHO estimate, in India, around 40 million people are chronic HBV carriers [3].Among 26 million infants born every year in India, approximately 1 million develop chronic HBV infection [6]. In India each year, around one million people die from HBV induced liver diseases, which equates to about two HBV-related deaths per minute. Moreover, due to the presence of wide geographical variations, different socioeconomic status, religion and cultures the estimated prevalence of HBsAg in the tribal population of India, is 3.1% and in non-tribal population, it is 11.85% [7]. Based on the prevalence of HBsAg, different areas of the world are classified as having low (<2%), intermediate (2-7%) and high (≥8%) HBV endemicity [8]. Countries which have high endemicity include South-East Asia, China, most of Africa, most of Pacific Islands, the Amazon basin and parts of the Middle East, countries with intermediate endemicity include South Asia, Eastern and Southern Europe, Russia and Central and South America and low endemicity areas include United States, Western Europe, and Australia. India fall in South Asia has been grouped as a country with intermediate to high HBV endemicity [8].

The HBV is an enveloped partially double-stranded DNA virus that belongs to the hepatotropic DNA virus family (*hepadnaviridae*). It has the smallest genome of 3.2 kb among all DNA viruses [9]. This virus family has an evolutionary history as related virus reported to found in woodchucks, ground squirrels and ducks [9]. The infectious HBV virion (Dane particles) has a double-shelled spherical structure which is 42 nm in diameter and consisting of a lipid envelope containing HBsAg that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg) [4]. Nucleocapsid contains partially double-stranded DNA with two asymmetric strands. The plus strand of the HBV DNA is incomplete on the other hand minus strand or negative strand is complete but contains a nick at a unique site [10]. A negative strand of the DNA is not covalently closed due to the presence of viral polymerase on the 5' end of the strand [4]. With the help of electron microscopy, two types of particles were observed in the serum of infected individuals 42 nm to 47 nm Dane particles which determines the infectivity of the individual and 20 nm spherical subviral particles that do not contain the viral capsid [10, 11]. The Dane particles may range from as low as $10^2$ particles $mL^{-1}$ of serum in the occult and asymptomatic infections to more than $10^8$ particles $mL^{-1}$ during the active replicative phase of infection [10]. At the 5′ ends of both the strands short repeat sequence and direct repeats (DRs) of around 11 nucleotides present which play a vital role in priming viral replication [12]. Vaccination programmes for HBV effectively decreased the burden of the infection all over the world, but it is still a major public health concern in developing countries and various improvements are necessary in order to reduce the burden of this deadly disease [13]. Environmental factors, host genetic factors, immune responses, virus genotypes and viral load are the factors, which influence the susceptibility and progression of the disease [14, 15].

Various studies showed that HBV induces the autophagy process and use it for its own replication [16]. How HBV utilizes autophagy process for its own replication is still not clear [17]. A study showed that small surface protein (SHBs) of HBV enhances the autophagy process without promoting degradation of proteins via lysosome [17]. Other study demonstrated that HBV induces both autophagy and autophagic degradation [18]. Tang et al in their study demonstrated that HBx protein increases the autophagy process by up-regulating the expression of beclin-1[19]. HBx protein also inhibits the autophagic degradation by impairing lysosomal acidification that ultimately affects lysosomal degradative capacity [20]. In a study conducted by Tian and colleagues lower levels of HBV DNA, HBsAg and HBeAg were found in the liver-

specific Atg5 knockout mice, which shows that autophagy inhibition leads to a reduction in HBV replication [21].

Autophagy is an important cellular process that helps in degradation of dead cell organelles, unwanted proteins, in the elimination of various intracellular pathogens and protects cells from various stress conditions [22]. In the autophagy process, unwanted cell material is enclosed by isolating membrane or phagophore that form double-membrane vesicle called as autophagosome, which delivers the cytoplasmic contents to the lysosome [21]. Autophagosome formation is an essential step and various ATG (autophagy-related) genes regulate this step. Until now in the yeast 40 autophagy-related proteins have been identified, among them, 18 proteins are involved in autophagosome formation [23, 24]. Alteration in autophagy pathway genes and proteins has been well documented in the implications of various diseases such as neurodegenerative diseases, cardiovascular disorders, cancer, aging and other inflammatory diseases and contributes to the intervention of novel therapeutic targets [25, 26]. Modulation in autophagy process has already shown better results in the treatment of various diseases such as cancers, Alzheimer's and Parkinson's diseases and many others [27].A number of polymorphisms in various genes such as MHC class I loci and class II loci, cytokine, chemokine and vitamin D receptor have been tested for their associations with HBV persistence and disease outcomes. Various polymorphisms in genes Th17, HLA-DP, IL-28B and eIF-2a are found to be associated with sustained responses to pegylated interferon's treatment [28-30]. Identification of various polymorphisms of the genes those directly or indirectly play role in the HBV infection, treatment and vaccination would be helpful in developing more effective treatments regimens and preventive measures [31]. From the above discussion, it is clear that various polymorphisms of many genes play role in the pathogenesis of HBV infection, but so far, no such polymorphisms in the autophagy-related genes have been studied for their association with HBV infection susceptibility and outcome. HBV virus uses the autophagy process to enhance its replication, hence indicating the possibility of targeting the pathway for the treatment of HBV infection [32].

A recent study demonstrated the increased expression of *ATG16L1* gene (an autophagy gene) in HepG2 and HepG2.215 cell lines (cell lines infected with HBV genome) in comparison with THLE-2 cell line (normal liver cell line) suggesting its role in HBV infection [33]. *ATG16L1* protein makes a complex with the ATG12–ATG5 conjugate that helps in the recruitment of LC3

to the developing phagophore [34]. The ATG12–ATG5/ATG16L1 complex is a ~800-kDa protein that dissociates from the completed autophagosome [35]. Defects in *ATG16L1* gene affect the formation of autophagosome, degradation of long-lived proteins and clearance of bacteria [36]. Atg16l1 protein mainly consists of three domains, N-terminal domain where ATG5 binds, a central coiled-coil domain that helps in self-oligomerization and WD repeats C domain [35]. WD domain provides docking stage for an amino acid motif that present in the intracellular region of TMEM59 (transmembrane protein) and other proteins that lead to increase in autophagy process (xenophagy) by the activation of LC3 lipidation [37]. Overexpression of *ATG16L1* was found to be associated with various diseases such as oral squamous cell carcinoma disease, Crohn's disease and colorectal cancer [33]. *ATG16L1* gene polymorphisms found to be associated with various diseases such as Crohn's disease, inflammatory bowel disease, psoriasis vulgaris, palmoplantar pustulosis, a Paget disease of bones and gastric cancer[38-44]. Another important autophagy gene, *IRGM* helps in the initiation of autophagy pathway and it is the most commonly targeted protein by RNA viruses [45]. *IRGM* interacts with various other autophagy pathway proteins such as *ATG5*, *ATG10*, *MAP1CL3C* and *SH3GLB1*, ULK1 and Beclin 1 which regulates earlier steps of the pathway, suggesting its role in nucleation or elongation step of autophagy [46, 47]. These proteins along-with *IRGM* regulate the initial steps of the autophagosome formation and viral infection modulates this interaction, which in turn modulate the autophagy process [46]. Role of polymorphisms of *IRGM* gene has already been well documented in the pathogenesis of many diseases such as inflammatory bowel disease, tuberculosis, and many others [48-53]. Defects in the autophagy pathway have been implicated in various human disease, hence it can be a viable target for drug discovery and to develop more effective therapeutic approaches for various diseases [54, 55]. Therefore, in this study, we have targeted polymorphisms of two important autophagy genes *ATG16L1 and IRGM* which are extensively studied in the implications of various diseases, and we have genotyped these SNPs in 550 HBV infected patients and 247 healthy control subjects to find out their association with HBV susceptibility.

## RATIONALE OF THE STUDY

Various polymorphisms of interferon-λ4, HLA-DPA1, HLA-DPB1, NTCP and IL-28 B genes have been found to be associated with HBV infection susceptibility and treatment response to the

drugs. Hepatitis B virus uses the autophagy process for its own replication. Increase in the autophagy process also leads to the increase in the levels of HBV DNA and vice-versa. This pathway could be targeted to develop more effective therapeutic approaches and to treat HBV infected individuals. In this study, we have targeted two important autophagy genes *ATG16L1* and *IRGM*, which help in initial steps in autophagy process, and any alteration of gene expression could lead to altered autophagy process and finally may predispose individuals differentially to HBV. We explored four polymorphisms of *ATG16L1* gene (rs2241880, rs2241879, rs13005285 and rs7587633) and three promoter region polymorphisms (rs4958842, rs4958843, and rs4958846) of *IRGM* gene. If the studied SNPs found to be associated with HBV infection, these could be used as biomarkers for risk analysis after validation them in a large cohort and after studying their impact on treatment response they can be used for developing more effective individualized therapies.

**Keeping in view the gaps the current study was undertaken with the following objectives**

**Objective 1:** To develop A-RFLP methods for genotyping promoter SNPs (rs4958842, rs4958843, rs4958846) of *IRGM*.

**Objective 2:** To genotype promoter SNPs in healthy controls and HBV infected patients and find their role in HBV susceptibility.

**Objective 3:** To develop A-RFLP (for rs13005285 and rs7587633) and RFLP (for rs2241879) methods for genotyping intronic and exonic SNPs of *ATG16L1*.

**Objective 4:** To genotype *ATG16L1* SNPs in healthy controls and HBV infected patients and to look for their genetic association in HBV susceptibility.

**Objective 1: To develop A-RFLP methods for genotyping promoter SNPs (rs4958842, rs4958843, rs4958846) of *IRGM*.**

*IRGM* gene polymorphisms that are associated with tuberculosis and Crohn's disease are one of the earliest known examples documenting the role of autophagy in human diseases [56]. Many polymorphisms including promoter region (rs4958842 (-1208 A/G), rs4958842 (-1161 C/T) and rs4958846 (-947 C/T) of the *IRGM* gene confer risk to leprosy, sepsis, gastric cancer, language impairment, Crohn's disease and tuberculosis and [48-53]. These studies have used direct sequencing, ligation detection reaction and tetra-primer ARMS-PCR for genotyping, which are expensive and need sophisticated infrastructure. Among these, all techniques for genotyping SNPs, restriction fragment length polymorphism (PCR-RFLP) is a simple, easy to perform and has been commonly used in laboratories to study genetic variations and mutations in complex diseases [57]. PCR-RFLP can be accomplished by digestion of either natural restriction enzyme site containing polymorphic site or by digestion of artificially created restriction site through mismatch of primers used for amplification [57, 58]. Because of the absence of restriction enzyme digestion site in the polymorphic position of *IRGM* promoter region SNPs the SNPs cannot be genotyped using PCR-RFLP, therefore, we developed artificial restriction fragment length polymorphism (A-RFLP) method for the SNP. The newly developed assays were applied to genotype samples from the normal healthy individuals. Therefore, we developed artificial restriction fragment length polymorphism (A-RFLP) methods to genotype these variations, which are simple, cost-effective and easy to perform.

**Methodology**

We have collected blood samples from 551 HBV infected and 247 healthy individuals. All the healthy control individuals included in the study had no previous history or family history of any genetic disorder or any other ongoing disease. All experiments were performed following guidelines of Institutional Ethical Committee of Jaypee University of Information Technology, Solan, Himachal Pradesh, India. Genomic DNA was isolated from the collected samples by inorganic salting out method given by Miller et al [59]. Quantity and quality of the isolated DNA were checked spectrophotometrically and by agarose gel electrophoresis, respectively [60] and stored at $-20^0$C until further use. Primers were designed by using Primer 3 software and manually

and in the forward primer, mismatches were introduced so that amplified PCR product contains restriction site (Table 1). A tail (of nucleotides A and C) of approximately 39 bp was also added in the forward primer to differentiate restriction enzyme digested products. Briefly genomic DNA for all three SNPs was amplified in 25 µl reaction mixture containing 0.6 U of Taq DNA polymerase (New England Biolabs), 0.2 mM of dNTPs, 0.12 µM of each primer; 40 ng of genomic DNA. The PCR cycling parameters were: Initial denaturation at $95^0$C for 3 minutes, followed by 35 cycles of denaturation at $95^0$C for 30 seconds, annealing at 66ºC(rs4958842), 59ºC (rs4958843) and 65ºC (rs4958846) for 40 seconds, extension at 68ºC for 30 seconds followed by final extension at 68ºC for 7 minutes. PCR products were then checked on 2% agarose gel electrophoresis. Initially, we have amplified 10 samples (healthy individuals) for each SNP and sequenced them to know their genotypes. After getting three genotypes for each SNP, these were further used for optimizing A-RFLP methods. To perform A-RFLP, amplified products were subjected to digestion with respective restriction enzymes. Ten µl PCR products were digested in 15 µl of the reaction mixture with 1U of each NruI (for rs4958842), NheI (for rs4958843) and SalI (for rs4958846) at 37ºC overnight. Digested products were then resolved on 3.5% agarose gel and visualized by ethidium bromide staining under UV light. Amplification with these primers results in the introduction of restriction sites of enzymes NruI (for -1208A/G when A is present), NheI (for -1161 C/T when C is present) and SalI (-947 C/T when C is present). **Figure 1** is a schematic representation of strategy followed for the development of A-RFLP methods for genotyping mentioned SNPs.

**Table 1: Primer sequences for *IRGM* promoter polymorphisms, nucleotide mismatches introduced in these, restriction enzymes used and digestion product size after RFLP.**

| Gene/SNPs | Forward Primer sequence with tail of A and C nucleotides at 5'end[a] | Reverse Primer sequence | Recognition site | Restriction enzyme | Product size (bp)[b] |
|---|---|---|---|---|---|
| rs4958842 (−1208 A/G) | *$A_{(19)}C_{(20)}$*AGCATGCTGGCAGCCCTCGC***G** | GGGCCGGATGGCTGCTCTGA | TCGCGA* | *Nru*I | AA-260, 58<br>AG-318, 260, 58<br>GG-318 |
| rs4958843 (−1161 C/T) | $A_{(19)}C_{(20)}$TCAGCCTTGGCGCCCAC**G**CTAG | CCCTCACTGCCAGGGGCCAT | GCTAGC* | *Nhe*I | CC-237, 57<br>CT-294,237,57<br>TT-294 |
| rs4958846 (−947 C/T) | *A(19)C(20)*AGAGCAGCCATCCGGC***GT***CGA | TGCACTCTTCAGCCCTTGGGC | GTCGAC* | *Sal*I | CC-240,56<br>CT-296, 240,56<br>TT-296 |

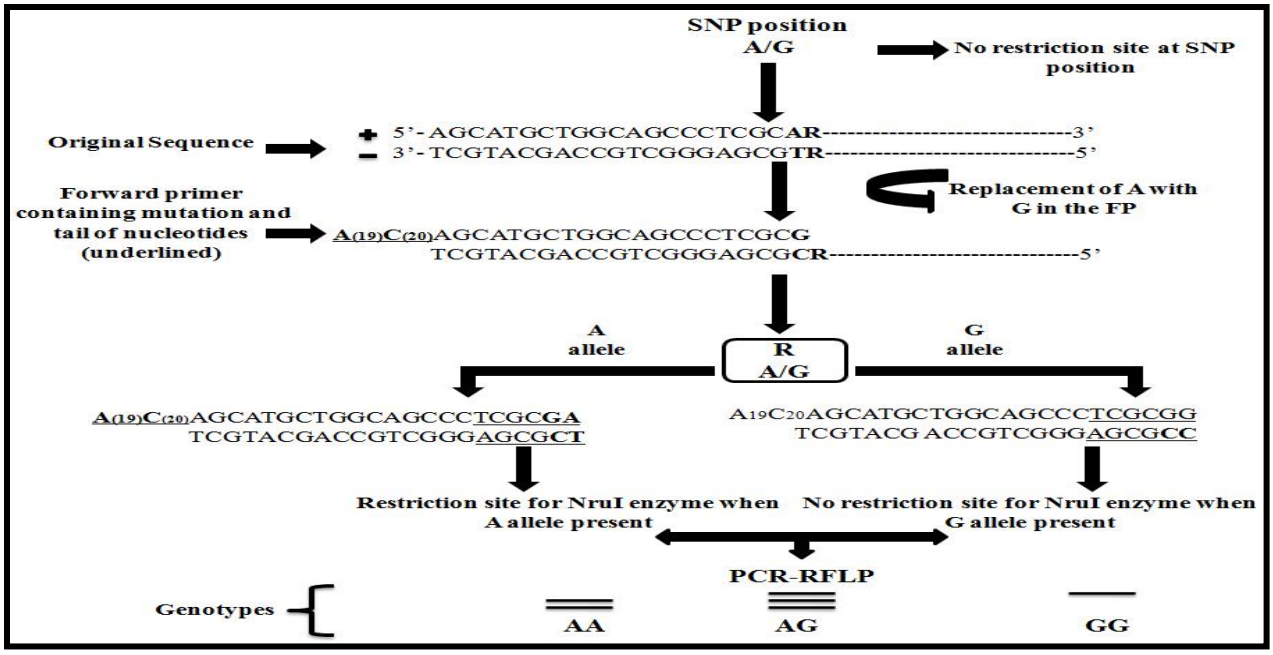


**Figure 1: Strategy followed for the development of Artificial-Restriction Fragment Length Polymorphism (A-RFLP) assay for the SNPs rs4958842.**

## Results

After sequencing 10 samples for each SNP we were able to get all three genotypes for rs4958842 (AA, AG and GG) and rs4958843 (CC, CT, and TT) while for rs4958846 we were able to get both samples with genotypes CT and TT. Results of newly developed A-RFLP method and sequencing were concordant. Further, the newly developed methods were used for genotyping the SNPs in all HBV infected and healthy control individuals. Figure 2 (a), (b) and (c) show chromatograms of sequenced samples and results of novel genotyping assays for rs4958842, rs4958843, and rs4958846, respectively.

## Conclusion

In conclusion, we have developed A-RFLP methods to genotype three *IRGM* promoter polymorphisms (rs4958842 (−1208 A/G); rs4958843 (−1161 C/T) and rs4958846 (−947 C/T), which is not present in any restriction enzyme sites. The developed method is easy, cost-effective, and reproducible. These methods would be helpful to study the role of these polymorphisms in various diseases and in various replication studies.

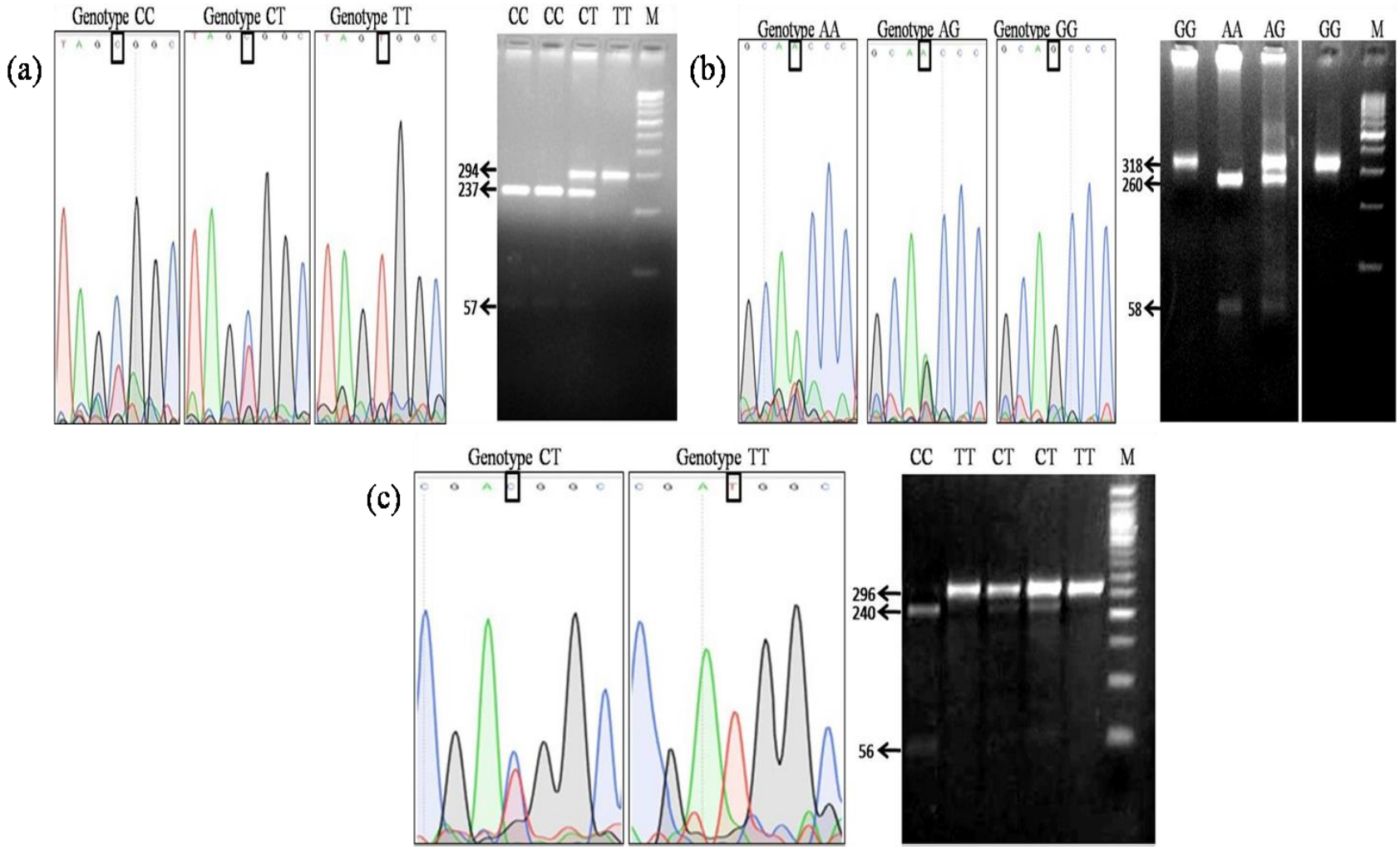


**Figure 2: Representative chromatograms of sequenced samples showing different genotypes and agarose gel electrophoresis of DNA samples genotyped by applying newly developed A-RFLP methods (a) rs4958842 (b) rs4958843 and (c) rs4958846. M in the gel indicates DNA marker and genotypes of the samples are shown on the top.**

**Objective 2: To genotype promoter SNPs in healthy controls and HBV infected patients and find their role in HBV susceptibility.**

Newly developed A-RFLP methods were further used to genotype three-promoter region SNPs of *IRGM* gene in HBV infected individuals and healthy subjects.

## Methodology

**Study population:** The study group comprised of 551 HBV infected subjects (220 Female, mean age 35.65±12.58 and 331 male, mean age 38.49±14.81) and 247 healthy individuals (108 Female, mean age 37.26±11.79 and 139 male, mean age 35.24±13.76) from North Indian Population. Demographic and clinical details of all the individuals enrolled in the study are summarized in **Table 2.**

**Table 2: Demographic and clinical characteristics of all subjects included in the study**

| Variable | Total HBV infected Individuals (n=551) | HBV Categories | | | | |
|---|---|---|---|---|---|---|
| | | Asymptomatic (n=111) | Acute (n=104) | CHB (286) | Cirrhosis (50) | Control (247) |
| **Female count** | 225(41%) | 41 (37%) | 44 (42%) | 123 (43%) | 17 (34%) | 129 (52.2%) |
| **Male count** | 326 (59%) | 70 (63) | 60 (58) | 163 (57%) | 33 (66%) | 118 (47.8%) |
| **Age (Yrs) Mean±SD** | 37.02±13.69 | | | | | |
| **HBsAg** | +ve | +ve | +ve | +ve | +ve | +ve |
| **HCV** | -ve | -ve | -ve | -ve | -ve | -ve |
| **HIV** | -ve | -ve | -ve | -ve | -ve | -ve |
| **ALT (IU/L)*** | 89±86.4 | 49.9±71.6 | 85±56.9 | 89.2±86.5 | 88±81 | 12±2.8 |
| **AST (IU/L)*** | 87.9±72.9 | 59±43.6 | 67.6±49 | 87.9±73.02 | 87.4±71.2 | 16±3.2 |

**Sampling and DNA isolation**: Venous blood was collected in vacutainers (with or without EDTA) from both infected and healthy subjects. Blood was carefully transported to the laboratory under the cold chain. Genomic DNA from blood samples was isolated using a standard inorganic method proposed by Miller et al [59] and stored at -20$^0$C.

**Assessment of biochemical parameters:** Serum of infected individuals was used for the identification of biochemical parameters such as Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT). AST and ALT levels were measured by using Erba Mannheim diagnostic kit.

**HBV DNA quantification:** We were interested to correlate HBV DNA levels with the various genotypes of polymorphisms. HBV DNA from serum samples of the patients was isolated using an Inorganic method described by Changotra et al [61] and the dried pellet was stored at −20 °C until further use. 380bp of HBV DNA was amplified and cloned into a pGEMT-easy vector for construction of standard curve. HBV DNA quantification was done with quantitative Real-Time PCR (using primers: forward and reverse primers used respectively were 5'-CCT GGY TAT CGY TGG ATG TGT-3' and 5'GGA CAV ACG KGC AAC ATA CCT T-3' and these primers amplify 116bp DNA fragment) protocol given by Roberta et al [62].

**Genotyping**: Genotyping of the all the three polymorphisms was carried out using newly developed A-RFLP methods [63, 64].

**Statistical analysis**

Odds Ratios (ORs) and confidence intervals (CI) for all the alleles and genotypes were obtained by logistic regression using Review Manager5.3 software. Studied SNPs were tested for Hardy-Weinberg equilibrium in the control population. Student t-test was used to compare continuous variables while Pearson chi-square analysis was used to compare categorical variables. Values were expressed as a mean ± standard deviation. An association was considered statistically significant if a p-value < 0.05 was attained. A 2-tailed p-value <0.05 was considered statistically significant. Comparisons between the genotypes, enzyme levels (AST, ALT) and HBV viral load levels were performed using the Student T-test.

**Results and Discussion**

Hardy-Weinberg equilibrium was assessed with HWE calculator in the control samples and genotypic frequencies of two SNPs (rs4958843 and rs4958846) were in accordance with Hardy-Weinberg equilibrium ($p>0.05$) but genotypes frequency in SNP rs4958842 deviate from HWE equilibrium ($p<0.05$). Figure 2 (a), (b) and (c) represents the gel pictures of genotyping for three SNPs. Association of the SNPs was tested using different genetic models (Allelic model, dominant model, co-dominant model and homozygous model) in five types of studied groups (HBV-control, Asymptomatic-control, Acute-control, CHB-control and Liver cirrhosis-control).

- **rs4958842 (G/A):** We found the protective role of SNP rs4958842 (G/A) in HBV infected individuals under genetic models: co-dominant (Odds Ratio (OR) =0.52; 95% confidence interval (CI) =0.38-0.71, $p< 0.0001$), dominant (Odds Ratio (OR) =0.51; 95%

confidence interval (CI) =0.38-0.70, p< 0.0001) and allelic (Odds Ratio (OR) =0.61; 95% confidence interval (CI) =0.48-0.78, p=0.0001). On analyzing allelic and genotypic frequencies in different categories of HBV individuals, we did not find any association in asymptomatic, acute and cirrhosis category. In individuals, with chronic infection we again found protective association in co-dominant (Odds Ratio (OR) =0.38; 95% confidence interval (CI) =0.26-0.54, p< 0.0001), dominant (Odds Ratio (OR) =0.52; 95% confidence interval (CI) =0.38-0.71, p< 0.0001), and allelic models (Odds Ratio (OR) =0.48; 95% confidence interval (CI) =0.35-0.65, p< 0.0001). The genotypic and allelic frequencies for the SNPs along-with Odds ratios and p values are given in **Table 3.** We did not find any difference in HBV DNA, AST and ALT levels in various infections stages and in the various genetic models.

- **rs4958843 (T/C):** Other promoter region SNP of *IRGM* gene rs4958843 (T/C) was found to be associated in disease susceptibility in chronic infection in co-dominant (Odds Ratio (OR) =1.52; 95% confidence interval (CI) =1.07-2.16, p= 0.01) and dominant models (Odds Ratio (OR) =1.41; 95% confidence interval (CI) =1.00-2.00, p=0.04). No association was observed in other stages of HBV infection. To evaluate if there is any correlation between genotypes and viral load we performed students T-test. A significant difference in viral load was observed only in the acute category. AST and ALT levels were also compared with the three genotypes obtained but no significant difference was observed.
- **rs4958846 (T/C):** SNP rs4958846 found to play protective role in HBV infection under genetic models: allelic (Odds Ratio (OR) =0.73; 95% confidence interval (CI) =0.59-2.00, p=0.04), homozygous (Odds Ratio (OR) =0.42; 95% confidence interval (CI) =0.24-0.74, p=0.0003) and dominant (Odds Ratio (OR) =0.72; 95% confidence interval (CI) =0.53-0.98, p=0.04). We also found the protective association of the SNPs in chronic and cirrhosis categories under allelic, co-dominant, dominant and homozygous models. No significant difference was observed in HBV DNA levels, AST and ALT in different genotypes.

**Table 3: Genotypes and allele distribution of *IRGM* SNP rs4958842 in healthy controls and different categories of hepatitis B infection**

| Polymorphisms<br>rs4958842<br>G/A<br>MAF( A)==0.2037/1020 | HBV<br>(n/N[%])<br>(N=551) | Groups<br>Asymptomatic Carriers<br>(n/N[%])<br>(N=111) | Acute Carriers<br>(n/N[%])<br>(N=104) | CHB<br>(n/N[%])<br>(N=286) | Cirrhosis<br>(n/N[%])<br>(N=50) | Control<br>(n/N[%])<br>(N=247) | HWE p value |
|---|---|---|---|---|---|---|---|
| GG | 348 (63) | 62 (56) | 58 (56) | 200 (70) | 28 (56) | 116 (47) | - |
| GA | 197 (36) | 48 (43) | 44 (42) | 83 (29) | 22 (44) | 126 (51) | |
| AA | 6 (1) | 1 (1) | 2 (2) | 3 (1) | 0 (0) | 5 (2) | |
| G | 893 (81) | 172 (77.5) | 160 (77) | 483 (84.5) | 78 (78) | 358 (72.5) | |
| A | 209 (19) | 50 (22.5) | 48 (23) | 89 (15.5) | 22 (22) | 136 (27.5) | |
| **HBV-Control** | **Odds Ratio (95%CI)** | | | **p value** | | | |
| GG | **1**[Ref] | | | | | | |
| GA | **0.52(0.38-0.71)** | | | **< 0.0001** | | | |
| AA | 0.40(0.12-1.34) | | | 0.14 | | | |
| **GA+AA** | **0.51(0.38-0.70)** | | | **< 0.0001** | | | |
| **G** | **1**[Ref] | | | | | | |
| **A** | **0.61(0.48-0.78)** | | | **0.0001** | | | |
| **Asymptomatic –Control** | | | | | | | |
| GG | **1**[Ref] | | | | | | |
| GA | 0.71(0.45-1.12) | | | 0.14 | | | |
| AA | 0.37(0.04-3.27) | | | 0.37 | | | |
| **GA+AA** | 0.69(0.44-1.09) | | | 0.12 | | | |
| **G** | **1**[Ref] | | | | | | |
| **A** | 0.76( 0.52-1.10) | | | 0.15 | | | |
| **Acute-Control** | | | | | | | |
| GG | **1**[Ref] | | | | | | |
| GA | 0.69(0.43-1.11) | | | 0.13 | | | |
| AA | 0.80(0.15-4.24) | | | 0.79 | | | |
| **GA+AA** | 0.70(0.44-1.11) | | | 0.13 | | | |
| **G** | **1**[Ref] | | | | | | |
| **A** | 0.78( 0.54-1.15) | | | 0.22 | | | |
| **CHB-Control** | | | | | | | |
| GG | **1**[Ref] | | | | | | |
| **GA** | **0.38(0.26-0.54)** | | | **< 0.0001** | | | |
| AA | 0.34(0.08-1.48) | | | 0.15 | | | |
| **GA+AA** | **0.38(0.26-0.54)** | | | **< 0.0001** | | | |
| **G** | **1**[Ref] | | | | | | |
| **A** | **0.48(0.35-0.65)** | | | **< 0.0001** | | | |
| **Cirrhosis-Control** | | | | | | | |
| GG | **1**[Ref] | | | | | | |
| GA | 0.72(0.39-1.33) | | | 0.30 | | | |
| AA | 0.37(0.02-6.91) | | | 0.50 | | | |
| **GA+AA** | 0.69(0.37-1.28) | | | 0.24 | | | |
| **G** | **1**[Ref] | | | | | | |
| **A** | 0.74( 0.44-1.23) | | | 0.25 | | | |

**Haplotype analysis:** Haplotype analysis of the *IRGM* gene was also done by SHEsis software. Haplotypes of three loci (rs4958842, rs4958843, and rs4958846 in the *IRGM* gene and their frequencies in case and control group are shown in Table 4. Haplotype analysis predicted seven haplotypes and all the haplotypes with a frequency lower than 3% were excluded. Among seven predicted haplotypes, only three haplotypes (CCGC, TCTC, and TTTC) found to be associated with HBV infection. The haplotypes CCGC (OR=0.497, 95%CI=0.325-0.759, p=0.001) and TCTC (OR=0.374, 95%CI=0.234-0.596, p=<0.0001) were appeared as protective factor against hepatitis B infection whereas haplotype TTTC appeared as a risk factor against hepatitis B infection (OR=7.071, 95%CI=(2.998-16.676), p= p=<0.0001).

**Table 4: Haplotype frequencies of rs4958842, rs4958843 and rs4958846) in *IRGM* gene in both cases and control groups**

| Haplotypes (rs4958842-rs4958843-rs4958846) | Case (freq) | Control(freq) | p-value | OR (95%CI) |
|---|---|---|---|---|
| A C C | 15.25(0.019) | 14.26(0.035) | 0.089379 | 0.535 (0.257-1.113) |
| A T C | 60.31(0.076) | 27.99(0.070) | 0.676432 | 1.104 (0.693-1.759) |
| A T T | **69.20(0.087)** | **66.99(0.167)** | **4.99e-005** | **0.479 (0.334-0.688)** |
| G C C | 31.65(0.040) | 23.54(0.059) | 0.150411 | 0.671 (0.388-1.160) |
| G C T | 106.86(0.135) | 51.44(0.128) | 0.725536 | 1.066 (0.746-1.523) |
| G T C | **138.79(0.175)** | **95.20(0.237)** | **0.011861** | **0.686 (0.511-0.921)** |
| G T T | **358.69(0.453)** | **117.81(0.293)** | **7.64e-008** | **2.016 (1.558-2.607)** |

## Conclusion

We have genotyped three-promoter region SNPs with the help of newly developed A-RFLP methods in HBV and healthy subjects. The gene *IRGM* variations rs4958842 and rs4958846 play role in protection from HBV infection, while rs4958843 found to play role in disease susceptibility in chronic infection. These could be used as prognostic biomarkers but definitely, these need to be confirmed further in a large number of samples in the follow-up replication studies.

**Objective 3: To develop A-RFLP (for rs13005285 and rs7587633) and RFLP (for rs2241879) methods for genotyping intronic and exonic SNPs of *ATG16L1*.**

The polymorphisms rs13005285, rs7587633, rs2241880, and rs2241879, spans a region of 36 kb within *ATG16L1* gene. These four SNPs are found to be associated with various diseases such as Crohn's disease, inflammatory bowel disease, psoriasis vulgaris, palmoplantar pustulosis, Paget disease of bones and gastric cancer[38-44].Two SNPs rs13005285 and rs7587633 do not contain restriction site of any restriction enzyme in order to perform genotyping by the PCR-RFLP method. We have developed A-RFLP methods to genotype these intronic variants. To genotype SNP rs2241879 by applying PCR-RFLP, we have designed primers with the help of Primer3 software and natural restriction site was present for the restriction enzyme Tsp45I. Genotyping of nsSNP rs2241880; A/G was carried out by the already standardized PCR-RFLP method described by Csöngei et al [39].

## Methodology

The methodology of the creation of restriction site has been well defined in the methodology section of Objective 1 of the study. Amplification with the mutated primers results in the creation of restriction sites for enzymes *Hind*III (for rs13005285) and *Eco*RI (for rs7587633) in the amplified product. Primer pairs and restriction enzymes used for the genotyping of rs2241880, rs2241879, rs13005285 and rs7587633 polymorphisms are mentioned in **Table 5.**

**Table 5: Primer sequences for amplification of target region for *ATG16L1* polymorphisms, nucleotide mismatches introduced in these, primer annealing temperatures, restriction enzymes used and digestion products sizes after RFLP.**

| Polymorphism | Primer Sequences [(a)] | Restriction Enzymes | Product Size (bp) |
|---|---|---|---|
| rs2241880 (A/G) | **FP:**CTCTGTCACCATATCAAGCGTGG<br>**RP:**TCTAGAAGGACAGGCTATCAACAGA | *SfaN*I | AG-282,110,172<br>GG-110,172<br>AA-282 |
| rs2241879 (G/A) | **FP:**TTTGCCCCATCCCTCAT<br>**RP:**ATTTCTTAGGAGACGCTCTG | *Tsp45*I | GA-366,211,155<br>GG-211,155<br>AA-366 |
| rs13005285 (G/T) | **FP**: $A_{(19)}C_{(20)}$AGCCCAGTGGAGCAGAGAAA**G**CT<br>RP: CCTCCTACAGGCATTCATTCACCTC | *HinD*III | GT-269,219,50<br>TT-219,50<br>GG-269 |
| rs7587633 (C/T) | **FP**: $A_{(19)}C_{(20)}$TAGGCTTGTGATGTTACTCAG**A**A**T**T<br>**RP**:CCAGAGATGACAATCAAGATAAGGAC | *EcoR*I | CT-467,416,51<br>CC-416,51<br>TT-467 |

**PCR reaction and restriction digestion:** To standardize the A-RFLP and PCR-RFLP reaction conditions for these four polymorphisms we have tried a different range of primers concentration, annealing temperature, and restriction enzyme. For all the polymorphisms DNA fragments were amplified in a 20µl reaction mixture containing genomic DNA concentration of 40 ng per reaction, 0.2 µl Taq polymerase (5U/µl; i-Taq TM DNA Polymerase), 2 µl Buffer (10X), 0.4 µl of dNTP's (10 mM) 0.25 µl of each primer (10 µM/µl); 15.9 µl of nuclease-free water. The PCR amplifications were conducted under following reaction conditions: Initial denaturation at $94^0$C for 3 min, followed by 35 cycles of denaturation at $94^0$C for 30 seconds, annealing for 40 seconds at $55^0$C (rs2241880), $50^0$C (rs2241879), $59^0$C (rs13005285) and $57^0$C (rs7587633) and extension at $72^0$C for 30 seconds each, and a final extension at $72^0$C for 7 minutes. Amplified PCR products were analyzed on 2% ethidium bromide-stained agarose gel. For Restriction fragment length polymorphism amplicons were digested with 0.5 units of *SfaN*I (rs2241880), 0.5 units of *Tsp45*I (rs2241879), 1 unit of *Hind*III (rs13005285) and 1 unit of EcoRI (rs7587633). The restriction fragments were analyzed by electrophoresis on 2.5% and 3% agarose gel containing ethidium bromide and visualized under UV light. Banding patterns observed after PCR-RFLP for all the four SNPs are mentioned in Table 5.

**Results:** We have developed A-RFLP methods to genotype two intronic variants (rs13005285; G/T and rs7587633; C/T) and PCR-RFLP reaction conditions were successfully optimized for other exonic (rs2241880) and intronic (rs2241879) variants of *ATG16LI* gene. The developed method is easy, cost-effective, and reproducible. These methods would be helpful to study the role of these polymorphisms in various diseases including various replication studies. Figure 3 (a), (b) and (c) and d shows the banding pattern of PCR-RFLP for three SNPs.

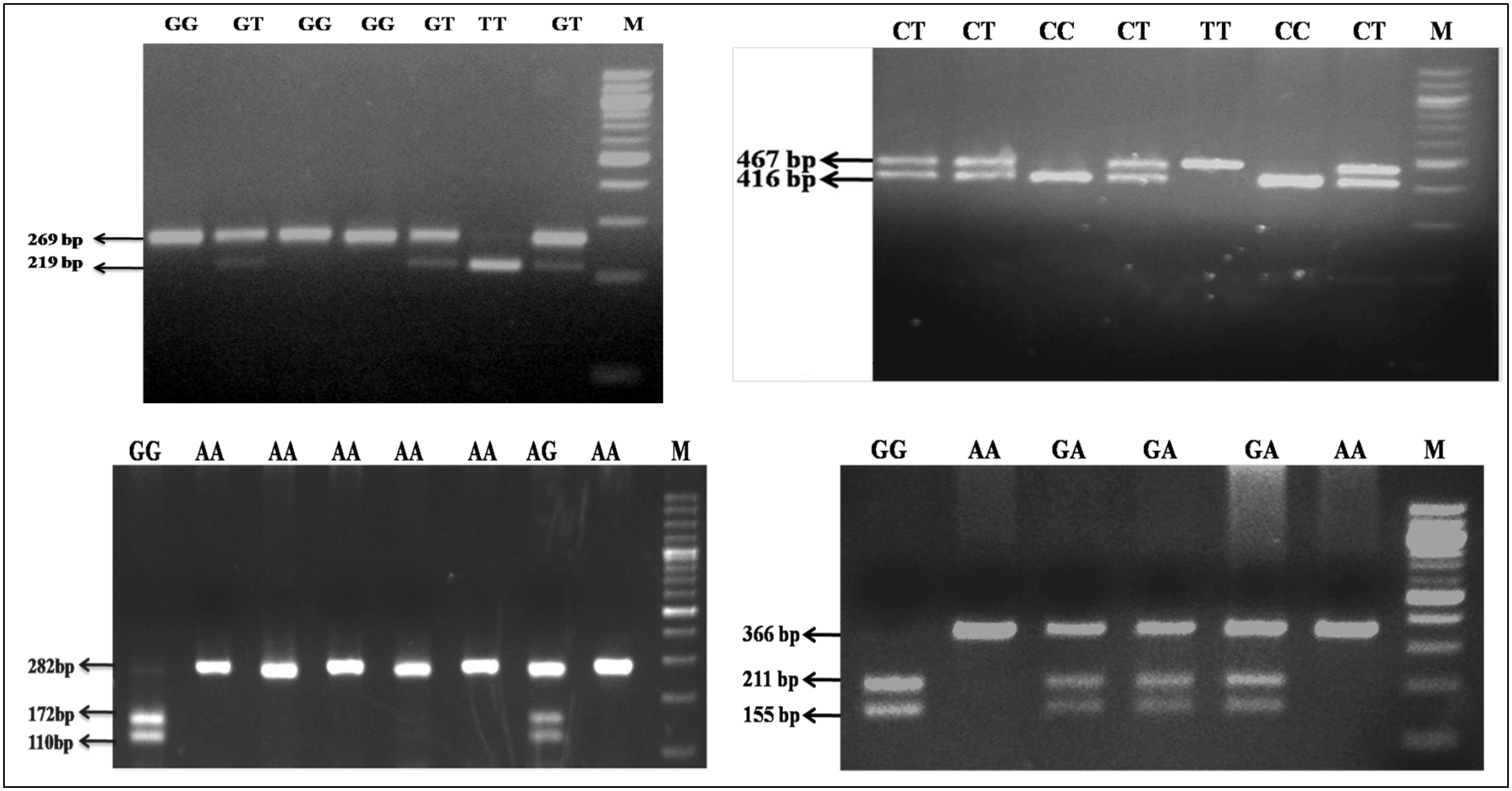


**Figure 3: Representative image for different genotypes obtained after PCR-RFLP analysis of *ATG16L1* SNPs (a) rs13005285 (G/T), (b) rs7587633 (C/T), (c) rs2241880 (A/G) and (d) rs2241879 (G/A). M in the gel indicates DNA marker and genotypes of the samples are shown on the top**

**Objective 4: To genotype *ATG16L1* SNPs in healthy controls and HBV infected patients and to look for their genetic association in HBV susceptibility.**

Newly developed A-RFLP and optimized PCR-RFLP methods were further used for the genotyping of these four variants in 551 HBV infected and 247 healthy control population.

**Methodology:** Various demographic and clinical parameters of the studied individuals and methodology used for the completion of this objective have been already shown in Table 2 (Methodology section of Objective 2).

**Results and Discussion**

Hardy-Weinberg equilibrium was assessed with HWE calculator in the control samples and genotypic frequencies of three SNPs rs2241879, rs13005285 and rs7587633 were in accordance with Hardy-Weinberg equilibrium ($p>0.05$) but genotypes frequency in SNP rs2241880 deviate from HWE equilibrium ($p<0.05$). Figure 3 (a), (b) and (c) and d shows the banding pattern of PCR-RFLP for three SNPs. Association of the SNPs were tested using different genetic models (Allelic model, dominant model, co-dominant model and homozygous model) in five types of studied groups (HBV-control, Asymptomatic-control, Acute-control, CHB-control and Liver cirrhosis-control).

**rs2241880 (A/G):** Our findings revealed that individuals carrying G allele found to increase the risk of HBV Infection in the studied population; Odds Ratio (OR) =1.31; 95% Confidence Interval (CI) = 1.06-1.63; P value=0.01. Allele G found to increase the risk of infection in asymptomatic category under various genetic models: allelic Odds Ratio (OR) =1.52; 95% CI = 1.10 - 2.08; p=0.0118, homozygous Odds Ratio (OR) =2.30; 95% CI = 1.22 – 4.36; p=0.01. Protective association was observed in chronic group under allelic model Odds Ratio (OR) = 1.36; 95% CI = 1.06 - 1.73; p=0.012 and homozygous model Odds Ratio (OR) =2.07; 95% CI = 1.22 – 3.53; p=0.0075. In case of acute and cirrhosis stages of infection, this SNP does not play role in the susceptibility to this infection. The genotypic and allelic frequencies for the SNP along-with Odds ratios and p values are given in Table 6. To evaluate if there is any correlation between genotypes and viral load we performed students T-test. The correlation between the polymorphism rs2241880 and the HBV viral load ($log_{10}$) showed higher viral load in the mutant GG genotype in asymptomatic and

chronic categories. AST and ALT levels were also compared with the three genotypes obtained but no significant difference was observed.

**rs2241879 (G/A):** On comparing frequencies of wild-type and mutant alleles of SNP rs2241879 in infected and normal individuals, we observed A allele predispose individuals to HBV infection under allelic Odds Ratio (OR) = 1.56; 95% CI = 1.26 - 1.94; p< 0.0001 and homozygous models Odds Ratio (OR) = 2.58; 95% CI = 1.59 - 4.18; p= 0.0001. On analyzing data of different stages of HBV infection, allele A also found to increase the risk of infection in chronic and cirrhosis stages of infection under allelic, homozygous and dominant models. A significant difference in viral load was observed in different categories but high viral load ($log_{10}$) was found to be associated with mutant genotype only in the chronic category of individuals. On comparing biochemical parameters, AST and ALT with various genotypes we did not did not find any significant association.

- **rs13005285 (G/T):** Similarly, SNP rs13005285 was also found to be associated with HBV susceptibility under genetic models: allelic Odds Ratio (OR) =1.39; 95% CI = 1.09 - 1.77; p= 0.0080, homozygous Odds Ratio (OR) =2.26; 95% CI = 1.13 - 4.50; p= 0.019 and dominant Odds Ratio (OR) =1.42; 95% CI = 1.05 - 1.93; p= 0.02. Allele T also found to increase the risk of infection in asymptomatic, acute and cirrhosis stages of infection under allelic, homozygous and dominant models. A significant difference in viral load was observed in asymptomatic and chronic categories but high viral load ($log_{10}$) was found to be associated with wild genotype. A significant difference in the mean values of ALT was observed in an asymptomatic category only. No significant difference was observed on comparing AST and ALT with various genotypes in other categories.

- **rs7587633 (C/T):** The mutant allele of the SNP rs7587633 (C/T) does not predispose individuals to HBV infection Odds Ratio (OR) =0.9228; 95% CI= 0.7064 - 1.2054; p =0.5556. On analyzing data of different categories of HBV infection, we did not find any significant association of allele T in the disease susceptibility or in protection. Furthermore, in genetic models, we found the protective role of the polymorphism in asymptomatic and cirrhosis categories under dominant and co-dominant genetic models. A significant difference in viral load was observed in chronic and cirrhosis categories and high viral load

($log_{10}$) was found to be associated with mutant TT genotype. No significant difference was observed in the mean values of AST and ALT with different genotypes.

**Table 6: Genotypes and alleles frequencies of *ATG16L1* rs2241880 (A/G) in HBV (that includes different stages) and in healthy controls**

| **Polymorphisms**<br><br>**rs2241880**<br>**A/G**<br>**MAF(G)=0.3960/1983** | **HBV (n/N[%]) (N=551)** | **Groups** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Asymptomatic Carriers (n/N[%]) (N=111)** | **Acute Carriers (n/N[%]) (N=104)** | **CHB (n/N[%]) (N=286)** | **Cirrhosis (n/N[%]) (N=50)** | **Control (n/N[%]) (N=247)** | **HWE p value** |
| AA | 125 (23) | 26 (23.4) | 33 (33.9) | 55 (19.2) | 11 (22) | 57 (23.1) | 0.0003 |
| AG | 266 (48) | 44 (39.7) | 47 (41.5) | 153 (53.5) | 22 (44) | 151 (61.1) | |
| GG | 160 (29) | 41(36.9) | 24 (24.6) | 78 (27.3) | 17 (34) | 39 (15.8) | |
| A | 516 (47) | 96 (43.2) | 113 (54.3) | 263 (46) | 44 (44) | 265 (53.6) | |
| G | 586 (53) | 126 (56.8) | 95 (45.7) | 309 (54) | 56 (56) | 229 (46.4) | |
| **HBV-Control** | **Odds Ratio (95%CI)** | | | **p value** | | | |
| AA | **1**[Ref] | | | - | | | |
| AG vs. AA | 0.82 (0.56 - 1.19) | | | 0.29 | | | |
| GG vs. AA | **1.96 (1.22 - 3.16)** | | | **0.005** | | | |
| AG+GG vs. AA | 1.02 (0.72-1.46) | | | 0.90 | | | |
| A | **1**[Ref] | | | - | | | |
| G vs. A | **1.31 (1.06 - 1.63)** | | | **0.01** | | | |
| **Asymptomatic –Control** | | | | | | | |
| AA | **1**[Ref] | | | - | | | |
| AG vs. AA | 0.64 (0.36 - 1.13) | | | 0.13 | | | |
| GG vs. AA | **2.30 (1.22 - 4.36)** | | | **0.01** | | | |
| AG+GG vs. AA | 0.98 ( 0.58 - 1.67) | | | 0.94 | | | |
| A | **1**[Ref] | | | - | | | |
| G vs. A | **1.52 (1.10 - 2.09)** | | | **0.01** | | | |
| **Acute-Control** | | | | | | | |
| AA | **1**[Ref] | | | - | | | |
| AG vs. AA | **0.54 (0.31 - 0.92)** | | | **0.02** | | | |
| GG vs. AA | 1.06 (0.55 - 2.06) | | | 0.86 | | | |
| AG+GG vs. AA | 0.64 (0.39 - 0.07) | | | 0.09 | | | |
| A | **1**[Ref] | | | - | | | |
| G vs. A | 0.97 (0.70 - 1.35) | | | 0.8683 | | | |
| **CHB-Control** | | | | | | | |
| AA | **1**[Ref] | | | - | | | |
| AG vs. AA | 1.05 (0.68 - 1.62) | | | 0.83 | | | |
| GG vs. AA | **2.07 (1.22 - 3.53)** | | | **0.0075** | | | |
| AG+GG vs. AA | 1.26 ( 0.83 - 1.91) | | | 0.28 | | | |
| A | **1**[Ref] | | | - | | | |
| G vs. A | **1.36 (1.07 - 1.73)** | | | **0.01** | | | |
| **Cirrhosis-Control** | | | | | | | |
| AA | **1**[Ref] | | | - | | | |
| AG vs. AA | 0.76 (0.34 - 1.66) | | | 0.48 | | | |
| GG vs. AA | 2.26(0.95 - 5.34) | | | 0.0636 | | | |
| AG+GG vs. AA | 1.06(0.51 - 2.21) | | | 0.86 | | | |
| A | **1**[Ref] | | | - | | | |
| G vs. A | 1.47 (0.95 - 2.27) | | | 0.08 | | | |

**Haplotype analysis:** Haplotypes of four loci (rs2241880, rs2241879, rs13005285 and rs7587633) in the *ATG16L1* gene and their frequency in cases and controls are shown in Table 7. Haplotype analysis predicted twelve haplotypes and all the haplotypes with a frequency lower than 3% were excluded. Among twelve predicted haplotypes, only three haplotypes (GGGC, AGTC, and AATC) found to be associated with HBV infection. The haplotypes GGGC (OR =0.497, 95%CI =0.325-0.759, p=0.001) and AGTC (OR=0.374, 95%CI=0.234-0.596, p<0.0001) were appeared as protective factor against hepatitis B infection whereas haplotype AATC appeared as a risk factor against hepatitis B infection (OR =7.071, 95%CI=(2.998-16.676), p<0.0001).

**Table 7. Haplotype frequencies of *ATG16L1* gene polymorphisms in cases and controls**

| Haplotypes | Case (freq) | Control (freq) | p-value | OR (95%CI) |
|---|---|---|---|---|
| G G G C | **47.33(0.061)** | **47.11(0.118)** | **0.001019** | **0.497 (0.325-0.759)** |
| G G T C | 36.10(0.047) | 14.76(0.037) | 0.400535 | 1.303 (0.702-2.418) |
| G A G C | 172.75(0.223) | 76.31(0.191) | 0.145814 | 1.252 (0.925-1.695) |
| G A G T | 34.47(0.045) | 25.18(0.063) | 0.197539 | 0.707 (0.416-1.201) |
| G A T C | 51.37(0.066) | 19.16(0.048) | 0.180385 | 1.444 (0.841-2.477) |
| A G G C | 91.20(0.118) | 42.96(0.107) | 0.519717 | 1.135 (0.772-1.669) |
| A G G T | 20.47(0.026) | 18.32(0.046) | 0.089448 | 0.576 (0.303-1.096) |
| A G T C | **33.76(0.044)** | **44.18(0.110)** | **1.98e-005** | **0.374 (0.234-0.596)** |
| A G T T | 23.74(0.031) | 14.23(0.036) | 0.695341 | 0.875 (0.448-1.708) |
| A G G C | 119.52(0.154) | 54.75(0.137) | 0.351061 | 1.179 (0.834-1.668) |
| A A G T | 29.07(0.038) | 22.19(0.055) | 0.175839 | 0.677 0.384-1.194) |
| A A T C | **70.98(0.092)** | **5.77(0.014)** | **2.66e-007** | **7.071 (2.998-16.676)** |

## Conclusion

We have successfully genotyped three-promoter region SNPs of *IRGM* gene and four SNPs of *ATG16L1* gene n 550 HBV patients as well as in 250 healthy controls. In case of IRGM SNPs, we found the protective association of the SNP rs4958842 (G/A) with HBV infection. The SNPs rs4958842 (G/A) also has a protective association with Crohn's disease. The other SNP rs4958843 (T/C) was found to be associated with HBV infection risk only in chronic hepatitis B infected (CHB) patients. The third SNP of IRGM gene rs4958846 (T/C) also found to be associated with HBV protection in totality and in chronic and cirrhosis category patients.

The SNP rs2241880 (A/G) is responsible for the change of an amino acid Threonine with Alanine at 300 position. The SNP has been implicated as a risk factor in Crohn's disease. In our population, the SNP rs2241880 found to be associated with HBV infection risk. The other three SNPs of ATG16L1 gene are present in the intronic region of the gene. The SNP rs2241879 (G/A) also found to be associated with an increase in HBV infection risk. The SNP rs13005285 (G/T) was also found associated with increased susceptibility to HBV infection. The last intronic region SNP rs7587633 (C/T) of ATG16L1 gene was found to be associated with decreased susceptibility to HBV infection risk.

In nutshell, the study highlights the importance of autophagy genes SNPs in the pathogenesis of HBV infection. We have successfully genotyped seven SNPs of IRGM and ATG16L1 genes in a sample of North Indian Population. We found an association of all the SNPs in HBV infection susceptibility. Further studies on genotyping of these SNPs in different population and in more number of samples are needed to explicate the role of these SNPs in HBV infection. These could be used as prognostic biomarkers but definitely, these need to be confirmed further in a large number of samples in the follow-up replication studies.

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## List of Publications

**[1]** Sharma A and Changotra H (2017) Novel artificial restriction fragment length polymorphism methods for genotyping IRGM promoter polymorphisms. *Inflammatory Bowel Disease* 23(10): E52–E53 **(Impact Factor: 4.6; SCOPUS).**

**[2]** Sharma A and Changotra H (2017) Mutagenic primer based PCR-RFLP assay for genotyping IRGM gene promoter variant rs4958843 (C/T) *Journal of Clinical Laboratory Analysis*. doi: 10.1002/jcla.22346. **(Impact Factor: 1.52; SCOPUS).**

## Conference Abstracts

**[1] Sharma A,** Duseja A, Changotra H (2017) Association of IRGM promoter polymorphisms with hepatitis B virus infection in North Indian population. 42nd Annual Meeting of the Indian Society of Human Genetics & International Symposium on Trends in Human Genetic Research & Management, Indian Institute of Sciences, Bangalore, 2-4 March, 2017.

**[2] Sharma A,** Duseja A and Changotra H (2017) Association of IRGM gene variant rs4958842 with Hepatitis B infection in North Indian population. 20th Punjab Science Congress, IET Bhaddal, Ropar, Punjab, 7-9 February 2017.

**[3] Sharma A** and Changotra H (2017) Mutagenic primer based PCR-RFLP assays for genotyping of three-promoter region SNPs (rs4958842, rs4958843 and rs4958846) of IRGM gene. 20th Punjab Science Congress, IET Bhaddal, Ropar, Punjab, 7-9 February 2017.

**[4] Sharma A**, Duseja A and Changotra H (2015) Association of autophagy gene Atg16L1 variant rs2241879 and Chronic Hepatitis B in North Indian population. National Conference on Recent Trends in Biomedical Engineering, Cancer Biology, Bioinformatics and Applied Biotechnology (BECBAB-2015), Jawaharlal Nehru University, New Delhi, 28 November 2015.